SEED METABOLITES ALTER THE DEVELOPMENT OF ASERGILLUS ssp.

A Senior Thesis

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SEED METABOLITES ALTER THE DEVELOPMENT OF *ASPERGILLUS* SPP.

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Three species of the genus *Aspergillus* (*A.*)—*A. nidulans*, *A. parasiticus*, and *A. flavus*—are currently being observed in our lab to determine the effects of seed metabolites on fungal development. *A. nidulans* reproduces asexually through the formation of conidiophores and sexually through the formation of cleistothecia. Both *A parasiticus* and *A. flavus* reproduce asexually via the conidiophore. The development of these reproductive structures seems to be partially dependent upon the fungal molecule called psi factor that is derived from the polyunsaturated fatty acid, linoleic acid. Psi factor is a hormone that controls sexual and asexual reproduction in the fungus. Due to the structural similarities and similar effects on fungal development, we hypothesize that these seed metabolites are behaving in the same manner as the *Aspergillus* psi factor and in this way are directing fungal development. The relationship between fungal development and production of the carcinogenic compounds aflatoxin and sterigmatocystin by *Aspergillus* has been recently demonstrated in our lab (Hicks et al., 1997). Therefore, understanding the effects of natural fatty acids on fungal development could aid in the reduction of these mycotoxins.

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INTRODUCTION

Aspergillus fungi are agriculturally and economically important plant pathogens. As part of their normal development, these fungi have a biosynthetic pathway that synthesizes two toxic chemical compounds—aflatoxin and sterigmatocystin. These mycotoxins can cause liver cancer in both humans and animals when ingested at certain levels from contaminated oilseed crops such as corn, peanuts, or cottonseed. The agriculturally important mycotoxin, aflatoxin, is a severe and, unfortunately a common problem in Texas especially for corn farmers who have faced three epidemics of aflatoxin contamination in the last six years. This fungus poses a very real threat to production agriculture, and it is a problem we eventually hope to solve.

The development and infection processes of the *Aspergillus* fungus are an important part of its disease cycle. The *Aspergillus* disease cycle begins with the production of conidia. Conidia, small, circular asexual spores produced by the fungus, are the causal agents of infection and, therefore, constitute the primary source of inoculum. The spores can be blown by the wind or carried by water to reach their host. The conidia enter its host seeds via breaks in the outer seed coating formed as a result of insect damage or cracking under moisture-stressed conditions. Under favorable growing conditions, the asexual spores begin to grow vegetatively by elongating into long, thin filaments known as hyphae. These hyphae continue to grow between the individual cells of the host seed, and eventually mature into a reproductive structure known as a conidiophore. The conidiophore releases the asexual spores, or conidia, which can initiate the disease cycle again. This cycle can occur many times in one growing season, and it is what often allows the fungus to infect entire fields and cause epidemics of

aflatoxin contamination. Epidemics can only occur when there are many repetitions of the asexual cycle during the growing season.

When conditions are no longer favorable for the fungus to continue this rapid reproduction, the fungus enters an over-wintering stage by producing either sclerotia or cleistothecia.[1] These darkly pigmented structures are larger and able to survive much harsher conditions than the conidia. When the unfavorable conditions pass, the disease cycle resumes with the maturation of the over-wintering structure into many conidiophores that produce more conidia.

This research is focused on determining what signals are required for the fungus to produce the asexual spores necessary for initiating the *Aspergillus* disease cycle. Previous research in our lab has shown that certain seed fatty acids can alter the amount of mycotoxin synthesized by the fungus (Burow et al., 1997). We also have shown that asexual reproduction is genetically linked to mycotoxin production (Hicks et al., 1997). Therefore, we followed these initial clues and hypothesized that seed fatty acids may also be affecting fungal development, specifically asexual sporulation.

The specific fatty acids which affected mycotoxin production are linoleic acid and two of its derivatives, 9-hydroperoxy linoleic acid, 9-HPODE, and 13-hydroperoxy linoleic acid, 13-HPODE, formed via a plant lipoxygenase (LOX) enzyme. The structure of these fatty acids is very similar to *Aspergillus*' own developmental signal known as psi factor, a derivative of linoleic acid and formed via a fungal LOX enzyme (Fig. 1). Psi

[1] Sclerotia are sterile over-wintering structures produced by asexual *Aspergillus* spp. such as *A. flavus* and *A. parasiticus*--two aflatoxin producers. Cleistothecia, containing sexual spores called ascospores, are the over-wintering structures produced by sexual *Aspergillus* spp. such as *A. nidulans*--a sterigmatocystin producer. These two structures are thought to be analogous, but there is no formal proof of this relationship.

factor is made of three components (PsiA, PsiB, and PsiC) and serves as a fungal sporulation factor that directs the fungus in producing reproductive spores. The similarity in structure of the fungal and plant compounds has led us to test the hypothesis that seed fatty acids are acting as *Aspergillus* developmental signals in a manner similar to psi factor.

MATERIALS AND METHODS

Strains of *Aspergillus* spp. The *A. nidulans* strains used in this research to observe the effects of fatty acids on fungal development and measure sterigmatocystin and psi production were WIM-126, FGSC26, FGSC4, and TTA11. *A. parasiticus* SU1 and *A. flavus* 12S, 70S, 13 tox, and tan were used to study the effects of fatty acid metabolism on sporulation, formation of over-wintering structures, and aflatoxin production.

Growth medium. The medium (YGT) used to conduct these experiments consisted of 0.5% (w/v) yeast extract, 2% (w/v) glucose, and trace elements (Champe, et al., 1987). For solid culture, the media was solidified with 1.5% (w/v) agar.

Preparation of psi extracts. Liquid YGT (Champe et al., 1987) was inoculated with 2000 conidia ml^{-1} of WIM-145 and agitated at 250 rpm at 42°C. At 96 hr, the mycelial pellets were removed by filtration and the medium was extracted twice with equal volumes of ethyl acetate. The combined extracts were dried, and the residue was redissolved in a volume of ethyl acetate equal to one-fiftieth the volume of the extracted medium (Champe et al., 1987).

Bioassay of psi extracts. Ethyl acetate extracts were dried on 12.5 mm diameter filter paper discs and placed on the agar surface at the time of inoculation with 10^6 conidia ml^{-1} of FGSC4 or WIM-126. Cultures were incubated at 37°C in the light. Cleistothecium primordia were visualized by treatment of WIM-126 plates with a laccase chromogenic substrate (4-amino-2,6-dibromophenol and the coupling agent 3,5-dimethylaniline) which stains the primordia green (Hermann et al., 1983).

Bioassay of fatty acids. The fatty acids used in this bioassay were myristic (14:0), palmitic (16:0), oleic (18:1), ricinoleic (18:1), linoleic (18:2), linolenic (18:3), eicosatrienoic (20:3), arachidonic (20:4), and eicosapentaenoic (20:5) acids. We also used 13-HPODE, 9-HPODE, and 13-hydroxy linoleic acid (13-HODE), which are linoleic acid derivatives. These assays were carried out on solid YGT media. The fatty acids were dissolved in methanol and absorbed onto 12.5 mm diameter filter paper discs and placed on the agar surface that had been inoculated with 10^6 conidia ml^{-1} of the fungal strain. Cultures of *A. nidulans* were incubated at 37°C in both light and dark conditions. The *A. parasiticus* and *A. flavus* cultures were placed at 28°C in light and dark. All experiments were triplicated. Sample plugs with a 17mm diameter were cored around the discs from all of the above treatments. The interior 12.5 mm plug corresponding to the area covered by the paper disc was discarded. Both conidia and ascospores (released from crushed cleistothecia) present in the sampled area were counted using a hemacytometer. Sclerotia from the *A. flavus* and *A. parasiticus* samples were separated using Miracloth, and their dry weight was measured on a precision balance.

Evaluation of oxidative enzyme inhibitors. Four oxidative enzyme inhibitors including SKF 525-A (P450 inhibitor) and the following lipoxygenase inhibitors—salicylhydroxamic acid, propyl gallate, and nordihydroguaiaretic acid—were individually incorporated at concentrations of 4 uM, 40 uM, and 400 uM into YGT media before autoclaving. The solid media was then inoculated with *A. nidulans* strain WIM-126. All samples were triplicated in both the light and dark with sample cores from a uniform area of fungal growth being harvested as described previously. The number of conidia and ascospores present in the sample area were recorded.

Statistics. ANOVA and Fisher's LSD were used for the statistical analysis.

RESULTS

The most significant findings of this research demonstrates that linoleic acid, 9-HPODE, and 13-HPODE are increasing asexual development in *A. flavus* and *A. parasiticus*, and depending on concentration and which fatty acid, asexual or sexual development in *A. nidulans* strains. Other polyunsaturated fatty acids showed similar trends in altering fungal development; however, they were not statistically significant. We also confirmed Dr. Champe's work that psi factor extracts promoted sexual development in *A. nidulans* and furthermore, demonstrated that they increased asexual sporulation in *A. flavus*.

Effects of fatty acids on sexual and asexual sporulating *Aspergilli* grown in light versus dark conditions.

LIGHT. Linoleic acid (0.01 and 0.1 mg) increased the production of ascospores in *A. nidulans* whereas both 9-and 13-HPODE decreased ascospore numbers (Table 1).

Asexual production was decreased in low concentrations of linoleic acid and increased in high concentrations of 13-HPODE. The saturated and monounsaturated fatty acids tested had no effects on fungal development. *A. flavus* 12S, *A. flavus* 70S, and *A. parasiticus* SU1 greatly increased asexual sporulation in response to increasing fatty acid concentration (Table 3).

DARK. In the absence of illumination, ascospore production by *Aspergillus nidulans* tended to decrease significantly with increasing concentration of the 9-HPODE and 13-HPODE fatty acid treatments (Table 2). Again, low concentrations of linoleic acid decreased conidial production whereas the high concentration of 13-HPODE increased conidiation. The saturated and monounsaturated fatty acids tested had no effects on fungal development. Development of sclerotia increased in the *A. flavus* and *A. parasiticus* sclerotia producers (Table 4). The production of conidia also increased though to a lesser extent than in the light.

Effects of fatty acid concentrations on *Aspergillus flavus* 12S.

There was a statistically significant increase in asexual spore production in response to linoleic acid, 13-HPODE, and 9-HPODE fatty acid treatments. Neither oleic acid nor palmitic acid affected sporulation. 13-HPODE also statistically (P=0.05) decreased sclerotia production in this experiment (Table 5). A similar non-statistical trend was seen in the linoleic acid and 9-HPODE treatments.

Effects of oxidative enzyme inhibitors on sporulation.

The presence of a P450 inhibitor, SKF 525-A, and two lipoxygenase inhibitors, propyl gallate (PG) and nordihydroguaiaretic acid (NDGA), significantly decreased the production of asexual spores (Table 6). Simultaneously, these two lipoxygenase

inhibitors effectively increased the production of sexual spores by *A. nidulans* strain WIM-126. Another lipoxygenase inhibitor, salicylhydroxamic acid, did not affect conidia formation.

DISCUSSION

Based upon this research, we have determined that the polyunsaturated fatty acids linoleic acid, 9-HPODE, and 13-HPODE are acting as *Aspergillus* sporogenic factors whereas the monounsaturated and saturated fatty acids, oleic and palmitic acid, respectively, have no effect on fungal development. The production of spores was altered in a manner reminiscent to that of psi factor (Champe et al., 1987) where it is described that psiB and psiC increase sexual sporulation and psiA increases asexual sporulation in *A. nidulans*. A combination of all three factors—as in our prepared extract—favors psiB/psiC effects, thereby increasing sexual development in *A. nidulans*. We found the psi extract increased asexual sporulation in the asexual spp. *A. flavus* and *A. parasiticus*. In all cases, linoleic acid, 9-HPODE, and 13-HPODE increased asexual production in the sclerotial *A. parasiticus* and *A. flavus* strains. In *A. nidulans*, linoleic acid (0.01 mg) increased sexual spores (psiB/psiC effects), 9- and 13-HPODE decreased sexual sporulation at high concentrations, and 13-HPODE had the added effect of increasing asexual sporulation (psiA effect).

We also saw an effect of the polyunsaturated fatty acids on sclerotial production in the *A. flavus* and *A. parasiticus* sclerotial strains, but the nature of this effect was light dependent (Tables 3, 4, and 5). Specifically, sclerotial production increased in the dark but decreased in light. The effects of light and dark on the production of sclerotia (over-

wintering bodies) may be indicative of similar environmental changes occurring at the end of a growing season. As the fall and winter months get nearer, the days get shorter and darker and are no longer favorable for the fungus to continue its normal development. The fungus responds by producing more sclerotia until conditions are more favorable for its growth and development.

The synthesis of psi factor is predicted to require a lipoxygenase (Champe et al., 1987) or a P450 monooxygenase (Dr. Hal Gardner, personal communication) for one of the steps. If the fungus does need these enzymes to synthesize psi factor, then one would assume that their inhibition would block the psi pathway. This would result in an alteration of fungal development. The P450 inhibitor, SKF 525-A, grossly affected fungal growth and for that reason the results cannot be specifically interpreted for sporulation effects. However, the three LOX inhibitors did not appear to greatly affect fungal growth so we can cautiously say that at least two of these compounds (NDGA and PG) have psiB/psiC-like effects at increasing concentrations. However, as both chemicals are not specific for lipoxygenases, the evidence is not enough to say they are inhibiting a LOX enzyme in the psi pathway. More research must be initiated before we can determine the exact role, if any, of an *Aspergillus* lipoxygenase in the synthesis of psi factor.

Understanding the effects of natural fatty acids on fungal development may lead to innovative methods to prevent aflatoxin contamination in the field. The results of this study and other research in Dr. Keller's laboratory has led us to hypothesize that the fungus has developed a highly specialized interaction with its host plant to ensure its continued dissemination and survival as follows.

There are three key steps in this interaction. First of all, it has been well documented in fungal physiology literature that the *Aspergillus* fungus secretes a lipase when it infects a seed. This fungal lipase is an enzyme that releases fatty acids from the host plant cell. These fatty acids, including palmitic, oleic, and linoleic acids, are subsequently available for the fungus to use. Recent data also makes possible the second step of this interaction. These reports suggest that the plant pathogenic fungi induce a plant enzyme called Δ12 desaturase. This plant enzyme converts oleic acid into linoleic acid—thus providing an even more abundant source of available linoleic acid for the fungus.

The third aspect of this interaction has recently been documented in our lab. We have shown that *Aspergillus* induces a peanut seed lipoxygenase (Burow and Keller, unpublished data). This enzyme converts linoleic acid into hydroperoxy linoleic acid (9-HPODE or 13-HPODE). All of these points suggest that there is a remarkable dialogue occurring between the plant and the fungus where the fungus signals the plant to synthesize products that allow the fungus to continue producing asexual spores. The repetition of the disease cycle with the help of plant fatty acids allows the fungus to continue growing and providing a source of inoculum for its lasting survival.

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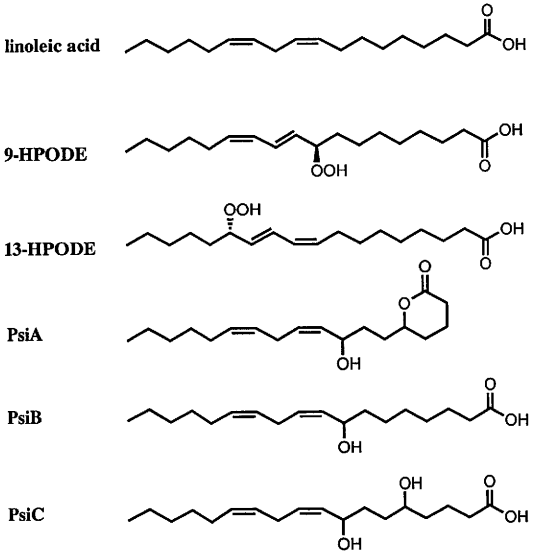
Figure 1. Chemical structure of linoleic acid and oxidized linoleic acids. 9-hydroperoxy linoleic acid, 9-HPODE, and 13-hydroperoxy linoleic acid, 13-HPODE, are two common products generated by plant lipoxygenases. PsiA, psiB, and psiC are linoleic-derived *Aspergillus* sporulation factors.



of different concentrations of linoleic acid (LA), oleic acid (OA), palmitic ,DE, and 13-HPODE on the number of conidia and ascospores of a 5-day *rgillus nidulans* strain WIM-126 grown in light.

Spores $x10^3/mm^2$

ıt	Conidia	Ascospores	Treatment	Conidia	Ascospores
			9-HPODE		
ntrol	157[a] AB	91 A	Control	225	120 A
.01 mg	53 C	170 B	0.01 mg	180	127 A
0.1 mg	121 B	160 B	0.1 mg	182	56 B
1.0 mg	181 A	80 A	1.0 mg	234	6 C
$LSD_{0.05}$[b]	45	58	$LSD_{0.05}$	ns	49
OA			**13-HPODE**		
Control	116	144	Control	424 AB	23 AB
0.01 mg	74	185	0.01 mg	283 A	56 A
0.1 mg	105	129	0.1 mg	253 A	49 A
1.0 mg	74	181	1.0 mg	583 B	0 B
$LSD_{0.05}$	ns	ns	$LSD_{0.05}$	229	37
PA					
Control	167	117			
0.01 mg	181	117			
0.1 mg	202	110			
1.0 mg	182	100			
$LSD_{0.05}$	ns	ns			

[a]Values are means of three replicates

[b]Means were analyzed by using Fisher's projected least significant differences at P=0.05.

Statistical differences are represented by different letters (A, B, C).

ns=not statistically significant.

TABLE 2. Effects of different concentrations of linoleic acid (LA), oleic acid (OA), palmitic acid (PA), 9-HPODE, and 13-HPODE on the number of conidia and ascospores of a 5-day culture of *Aspergillus nidulans* strain WIM-126 grown in the dark.

	Spores $\times 10^3/mm^2$				
Treatment	Conidia	Ascospores	Treatment	Conidia	Ascospores
LA			**9-HPODE**		
Control	64[a] BC	306	Control	42	353 A
0.01 mg	26 A	321	0.01 mg	62	226 B
0.1 mg	46 AB	271	0.1 mg	97	157 C
1.0 mg	95 C	255	1.0 mg	96	68 D
$LSD_{0.05}$[b]	37	ns	$LSD_{0.05}$	ns	68
OA			**13-HPODE**		
Control	28	340	Control	120 A	257 A
0.01 mg	13	238	0.01 mg	35 B	294 A
0.1 mg	21	322	0.1 mg	33 B	276 A
1.0 mg	9	303	1.0 mg	213 C	80 B
$LSD_{0.05}$	ns	ns	$LSD_{0.05}$	43	118
PA					
Control	46	256			
0.01 mg	52	182			
0.1 mg	61	178			
1.0 mg	61	199			
$LSD_{0.05}$	ns	ns			

[a]Values are means of three replicates

[b]Means were analyzed by using Fisher's projected least significant differences at P=0.05.

Statistical differences are represented by different letters (A, B, C).

ns=not statistically significant.

TABLE 3. Effects of different concentrations of linoleic acid on the number of conidia and sclerotia produced by 5-day cultures of *Aspergillus flavus* and *Aspergillus parasiticus* under illumination.

Treatment	Conidia[a] Spores x 10^3/mm^2	Sclerotia Dry weight (mg)	Treatment	Conidia Spores x 10^3/mm^2	Sclerotia Dry weight (mg)
***A. flavus* 12S**			***A. flavus* 13tox**		
Control	30975 A	6.3	Control	1398680	0
0.1 mg	108254 A	5	0.1 mg	1443387	0
1.0 mg	622700 B	3.6	1.0 mg	1350780	0
$LSD_{0.05}$[b]	165069	ns	$LSD_{0.05}$	ns	ns
***A. flavus* 70S**			***A. flavus* tan**		
Control	22992 A	4.1 A	Control	1382713	0
0.1 mg	319333 B	3.1 A	0.1 mg	1650953	0
1.0 mg	466227 C	2.7 B	1.0 mg	1606247	0
$LSD_{0.05}$	138493	0.8	$LSD_{0.05}$	ns	ns
***A. parasiticus* SU1**					
Control	472294 A	0.4			
0.1 mg	483790 A	0			
1.0 mg	661020 B	0			
$LSD_{0.05}$	124691	ns			

[a]Values are means of three replicates.

[b]Means were analyzed by using Fisher's projected least significant differences at P=0 05

ns=not statistically significant.

TABLE 4. Effects of different concentrations of linoleic acid on the number of conidia, sclerotia, and aflatoxin production of 5-day cultures of *Aspergillus flavus* and *Aspergillus parasiticus* grown in the dark.

Treatment	Conidia[a] Spores x 10^3/mm^2	Sclerotia Dry weight (mg)	Treatment	Conidia Spores x 10^3/mm^2	Sclerotia Dry weight (mg)
***A. flavus* 12S**			***A. flavus* 13tox**		
Control	2108 A	0.6 A	Control	62270 A	0
0.1 mg	3513 A	7.2 B	0.1 mg	277820 B	0
1.0 mg	90052 B	7.4 B	1.0 mg	248122 B	0
$LSD_{0.05}$[b]	9708	2.1	$LSD_{0.05}$	29935	ns
***A. flavus* 70S**			***A. flavus* tan**		
Control	1788 A	1.2 A	Control	489857	0
0.1 mg	5940 A	5.1 B	0.1 mg	922235	0
1.0 mg	49177 B	3.8 B	1.0 mg	840485	0
$LSD_{0.05}$	12586	2.5	$LSD_{0.05}$	ns	ns
***A. parasiticus* SU1**					
Control	46623 A	4.2			
0.1 mg	62270 A	4.1			
1.0 mg	127733 B	3.1			
$LSD_{0.05}$	33598	ns			

[a]Values are means of three replicates.

[b]Means were analyzed by using Fisher's projected least significant differences at P=0.05.

ns=not statistically significant.

TABLE 5. Effects of different concentrations of 13-HPODE, 9-HPODE, linoleic acid (LA), oleic acid (OA), and palmitic acid (PA) on the number of conidia, sclerotia, and aflatoxin production of a 5-day culture of *Aspergillus flavus* 12S under illumination.

Treatment	Conidia[a] Spores x 10^3/mm^2	Sclerotia Dry weight (mg)	Treatment	Conidia Spores x 10^3/mm^2	Sclerotia Dry weight (mg)
13-HPODE			**LA**		
Control	14562 A	4.3 A	Control	8750 A	3.4
0.1 mg	100271 B	3.7 B	0.5 mg	56522 B	3.3
1.0 mg	199903 C	3.5 B	1.0 mg	243971 C	2.6
$LSD_{0.05}$[b]	48128	0.6	$LSD_{0.05}$	30046	ns
9-HPODE			**OA**		
Control	16158 A	4.1	Control	6834	4.3
0.1 mg	85581 B	3.7	0.1 mg	10634	4.1
1.0 mg	307837 C	3.3	1.0 mg	11751	5.3
$LSD_{0.05}$	29764	ns	$LSD_{0.05}$	ns	ns
PA					
Control	6131	4.2			
0.01 mg	7025	4.9			
0.1 mg	7536	4.4			
$LSD_{0.05}$	ns	ns			

[a]Values are means of three replicates.

[b]Means were analyzed by using Fisher's projected least significant differences at P=0.05.

ns=not statistically significant

TABLE 6. Effects of different concentrations of lipoxygenase and P450 inhibitors on the number of conidia and ascospores of a 6-day culture of *Aspergillus nidulans* strain WIM-126 grown in light.

	Spores x 10^3/mm^2				
Treatment	Conidia	Ascospores	Treatment	Conidia	Ascospores
SKF 525-A (P450 inhibitor)			**PG**		
Control	806[a] A	55 AB	Control	806 A	55 A
4 uM	671 B	97 A	4 uM	662 B	85 A
40 uM	638 B	99 A	40 uM	753 AB	42 A
400 uM	147 C	7 B	400 uM	321 C	260 B
$LSD_{0.05}$[b]	103	51	$LSD_{0.05}$	97	67
SA			**NDGA**		
Control	806	55 AB	Control	806 A	55 A
4 uM	641	131 A	4 uM	646 B	69 A
40 uM	703	159 A	40 uM	527 C	79 A
400 uM	901	13 B	400 uM	168 D	240 B
$LSD_{0.05}$	ns	106	$LSD_{0.05}$	82	87

[a]Values are means of three replicates.

[b]Means were analyzed by using Fisher's projected least significant differences at P=0.05.

ns=not statistically significant.

SA=salicylhydroxamic acid.

PG=propyl gallate.

NDGA=nordihydroguaiaretic acid.